## INSTITUTO DE MEDICINA MOLECULAR JOÃO LOBO ANTUNES

# PREPARE SAMPLES FOR FLOW CYTOMETRY

**AUTHORS:** Andreia Santos
Mariana Fernandes
Rute Gonçalves

# SOP.UCF.005 – PREPARE SAMPLES FOR FLOW CYTOMETRY

## HOW DO YOU GET STARTED?

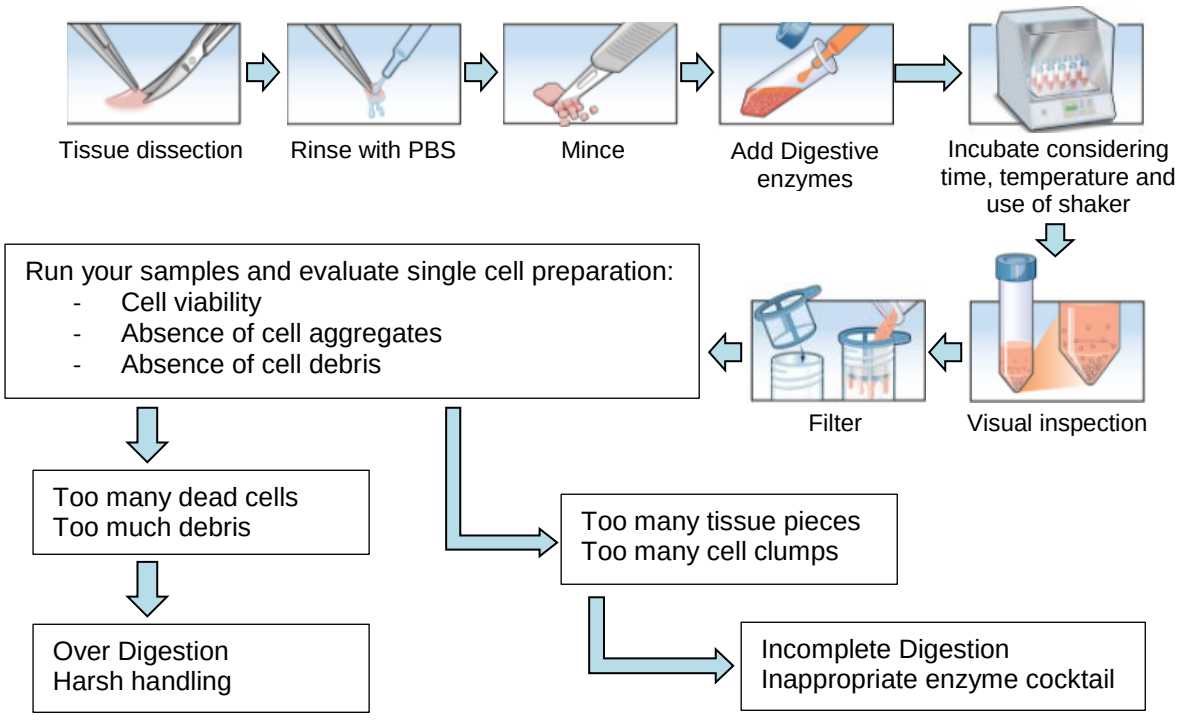
Talk to us about your experiment! If you want to use Flow Cytometry, we can help you design your new experiment. Get in contact with us and we will guide you through the Flow Cytometry Facility, and suggest the best systems for your project. Finally, we will book a training session with you and plan a trial run with your samples.

## HOW SHOULD YOU BRING YOUR SAMPLES?

### Obtain a Single-cell Suspension

Your cells must be in a single-cell suspension. Thus, peripheral blood cells or cells that grow in suspension are well suited for analysis by flow cytometry.

Adherent cell lines, solid tissue samples, and tumors require processing into single-cell suspensions before they can be analyzed. The protocol to get your samples into single-cell suspension may involve enzymatic digestion or mechanical dissociation of the tissue. Be careful when performing these protocols, as these may result in the destruction of the antibody epitope or cell damage.



The optimal cell concentration depends on how likely your cells are to clump together in the tube. These are the recommended concentrations:

- ***Flow Cytometer Analyzers:*** 1 million cells/mL, minimum volume 250 µL.
- ***Imaging Flow Cytometer:*** 20 million cells/mL, in a volume range of 15-200 µL.
- ***Cell Sorters:*** 50 million cells/mL (70µm nozzle), 20 million cells/mL (85 µm nozzle) and 5 million cells/mL (100 µm nozzle).

In all situations, removing cell clumps, dead cells, and debris is essential to eliminate false positives and obtain results of the highest quality.

After getting a single-cell suspension, you should bring your cells in the right buffer, to avoid reestablishment of cell clumps. A buffer of PBS + 2% FBS/BSA is a good basic buffer.

Please filter all your buffers with a 0.2 µm filter to reduce bioburden and microparticulate contamination.

Adding 25 mM HEPES buffer (pH 7.0) is a good idea as well, as HEPES has better buffering properties at high pressure than PBS does.

You may need to add 1mM EDTA, especially if you have adherent cells, as it helps chelate divalent cations that are often required for the formation of cell aggregates. In addition, if you have a high percentage of dead cells, adding DNase is strongly recommended as it reduces clumping caused by free DNA.

Before analysis or sorting, you should filter your cells through a nylon mesh. For analysis, a 70µm or 100µm mesh (BD Falcon™ cell strainers ref. 352350 and 352360) should work. For sorting, the mesh size should be inferior to the size of the nozzle: 40µm mesh for 70µm nozzle (BD Falcon™ cell strainers ref. 352340) and 70µm mesh for 85µm and 100µm nozzle.

# SOP.UCF.005 – PREPARE SAMPLES FOR FLOW CYTOMETRY

## Pay attention to the biosafety level of your samples

Notify us before bringing any cells of human or non-human primate origin to the core facility so we can help you assess the biosafety level of your cells. Most human primary cells and many human cell lines are BSL-2, posing hazards to laboratory staff and the environment.

BSL-1

Microbes are not known to cause disease in healthy hosts and pose minimal risk to workers and the environment

BSL-2

Microbes are tipically indigenous and are associated with diseases of varying severity. They pose moderate risk to workers and the environment

BSL-3

Microbes are indigenous or exotic and cause serious or potentially lethal diseases through respiratory transmission

BSL-4

Microbes are dangerous and exotic, posing a high risk of aerosol-transmitted infections, which are frequently fatal without treatment or vaccines. Few labs are at this level.

Only BSL-1 and BSL-2 samples are allowed in the UCF room. BSL-3 and BSL-4 samples are not permitted.

Also, many samples, such as clinical samples of COVID-19 patients, require additional enhancements to the standard BSL-2 work practices, such as the need of extra personal protective equipment (like safety goggles, extra gloves and KN95 safety masks) and the creation of a standard operation procedure (SOP) for ensuring compliance with the rules from all the staff members.

- **Analysis of cells by flow cytometry**: The analytical cytometers (*BD Accuri C6, BD LSRFortessa 2 / X-20, BD FACSymphony A5 SE, CYTEK Aurora and Amnis ImageStream Mark II*) are suitable for BSL-1 work only.

  BSL-2 cells must be fixed before use on these instruments. If your work requires analysis of unfixed BSL-2 cells, this can be done on the BD FACSAria Fusion and BD FACSymphony S6 SE with aerosol containment.
- **Sorting of cells by flow cytometry:** BSL-2 unfixed cells (e.g., human samples) may only be sorted on the BD FACSAria Fusion and BD FACSymphony S6 SE with aerosol containment. BD FACSAria III can only sort BSL-1 and fixed BSL-2 material. For all cells of human origin, provide product sheet listing biosafety level or proof the cells.

Many staining protocols include a fixation and/or permeabilization step. However, if yours doesn't and you need to fix your BSL-2 cells for analysis, we suggest a quick and easy PFA fixation (See Appendix A).

## Exclude your dead cells (cell viability staining)

Even though you keep your cells as happy as possible, some cells can die while handling and performing staining protocols. Dead cells can affect the analysis by compromising the integrity of the data by non-specifically binding antibodies. That's why most experiments benefit from adding a viability dye, to exclude dead cells from analysis and sorting. Also this viability staining can help you evaluate and compare the cell apoptosis and necrotic stages between cell conditions.

There are Fixable and Non-Fixable viability dyes. You can choose the right one for your assay depending on the purpose of your analysis and the post-staining protocol that will be performed. For more info see Appendix B.

## Perform the desired cell staining

If you are interested in analyzing or isolating specific cell subsets, you can label your cells with fluorochrome-conjugated antibodies or dyes.

- Cell surface markers can be used to define cell subsets based on lineage and developmental stage, as well as function. These surface markers have different forms and functions, including receptors for both soluble and cell-bound ligands, ion channels, glycoproteins, phospholipids, and more.
- Intracellular flow cytometry can be used to analyze a variety of intracellular molecules including cytokines, inflammatory mediators, transcription factors and phosphoproteins. It can provide you rich information about the function and signaling responses of specific cell subsets.
- DNA staining can be used for cell cycle analysis.

While staining of surface markers can be performed in live/unfixed cells, intracellular staining requires cell fixation and permeabilization before staining. Such fixation/permeabilization treatment allows the antibodies against intracellular antigens to cross the plasma membrane to stain intracellularly, while maintaining the morphological characteristic of cells.

Even when staining proteins inside the cell, it is important to consider their location as this may dictate the optimal protocol and buffer system. For example, the staining of nuclear proteins like Transcription

Factors works best with the strongest permeabilization protocol, while secreted proteins such as cytokines and chemokines work well best with a much softer permeabilization method.
For more detailed information see Appendix C

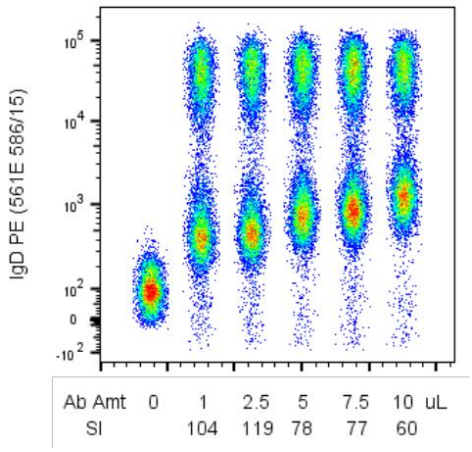
If you are using and antibody or fluorescent dye for the first time, please do a titration of that antibody/dye to access the preferable concentration to use in your samples. This is of extreme importance for optimizing resolution and obtaining robust results for population identification and expression level measurements.

## Antibody titration

Titration allows you to determine the amount of antibody that gives you the best separation of populations in your samples, and the best measure of expression levels. Too little antibody means the cells expressing the marker of interest do not stain as brightly as they could, and may not separate adequately from the negative cells. Too much antibody can increase non-specific binding, which increases the spread and background of the negative population. Both situations result in lower resolution of the measurement.

1. Titrate antibodies under the same staining conditions you will use in your experiment.
2. During the titration, each tube will contain only one antibody.
3. Test a range of antibody amounts above and below the amount recommended by the supplier. For example, for an antibody with a suggested volume of 5 µL per test, you might try 0.5, 1, 2.5, 5, and 7.5 µL. Include a sample with no antibody as well.
4. When acquiring data on the flow cytometer, be sure that the stained cells are on scale; look at the tube with the highest concentration of antibody for each titration before recording any samples.
5. Aim for 20,000 live, single cells in each data file. You might need more events if you are looking for a very rare population. It is important to have sufficient numbers of both positive and negative events. With only one antibody per tube, compensation is not necessary.
6. Check, for all concentrations tested, the separation between the positive and negative populations. It is best to aim for maximum separation, because spectral overlap from other colours may reduce resolution in the context of the full panel. Choose, also, the concentration that gives you a higher staining of the positive (not negative) population. This is translated in the calculation of the staining index for each concentration tested, as shown below.
   The selected Antibody should be the one with the highest SI.

$$Staining\ Index\ (SI) = \frac{Median_{Positive} - Median_{Negative}}{2 \times Standard\ Deviation_{Negative}}$$



## Choose the right tubes

- **Samples for the analyzers (BD LSRFortessa 2, X-20, BD FACSymphony A5 SE and CYTEK Aurora)** must be in BD Falcon™ Round-Bottom Tubes 5mL 12x75 mm polystyrene tubes (*ref. 352052* without cap, *ref. 352054* with cap, *ref. 352235* with filter-cap) or the identical but cheaper Sarstedt 5ml 75x12mm polystyrene tubes (*ref. 55.1579*).
- **For Amnis ImageStream Mark II**, it is only possible to use 1.5 mL tubes with a rectangular connection to the cap (Sarstedt *ref. 72.706*)
- **For BD Accuri C6**, you can use BD Falcon™ Round-Bottom Tubes 5 ml 12x75 mm polystyrene tubes (*ref. 352052* without cap, *ref. 352054* with cap, *ref. 352235* with filter-cap) or 1.5 mL tubes.
- **On the sorters BD FACSAria III, BD FACSAria Fusion and BD FACSymphony S6 SE** please use polypropylene tubes (Falcon 5 mL 12x75 mm (*ref. 352063*); BD Falcon™ Conical Tubes 15mL (*ref. 352096*) or 1.5 mL polypropylene tubes).

## WHICH CONTROLS DO YOU NEED?

Every experiment needs controls.

Bring unlabeled cells as a negative control. This sample will help you selecting the right cytometer settings for your acquisition. In addition, it will assist you in the analysis, while doing the gating strategy.

If you are using more than one color, you need single-color compensation controls. These will work to evaluate the spectral overlap between all the colors you have. If there is spillover between some colors, compensation will be performed.

Single-color controls can be cells or Anti-Ig k beads (for mouse, rat, hamster…). These beads can be helpful for compensation if you have minute samples, rare target populations or dim antigens. These are some examples:

- BD™ CompBeads (*ref.* 552843) are highly recommended for use in all experiments using tandem dye conjugates (e.g., PE-Cy™7, APC-Cy™7, etc.), which may have distinct spectral characteristics for each conjugate.
- OneComp eBeads™ Invitrogen™ (*ref.* 01-1111-41) are designed for use in compensation with all fluorochromes excited by blue (488 nm), green (532 nm), yellow-green (561 nm), and red (633-635 nm) lasers. This product is compatible with eFluor™ 450 but is not optimized for compensation of fluorochromes excited by a violet (405 nm) laser.

- Compensation Beads UltraComp eBeads™ Invitrogen™ (*ref.* 01-2222-42) are compatible with most standard lasers (UV to 633 nm) and improved for polymer dye use from violet laser.

While performing the Compensation setup, ensure you follow these principles:
- Compensation must be calculated with the same antibody/fluorochrome as the ones used in the final experiment;
- Bring the brightest markers for compensation;
- The positive and negative populations should have similar backgrounds.
In addition, you may need or want other controls for your experiment, such as FMOs.

## FOR SORTING, WHICH COLLECTION DEVICES CAN YOU USE?

You have many different options for collecting your sorted cells.

- Collect up to four different populations simultaneously into tubes of your choice, 1.5 mL, 5 mL tubes, or up to two population into 15 mL tubes.
- Sort into any kind of plate. 6-well, 12-well, 24-well, 36-well, 96-well, 384-well, PCR plate, etc.

The most recommended collection buffer is your cell culture medium with 10% FBS or some other serum. Collection tubes should be about 1/3 full of collection media.
We try to keep everything as clean as possible, but our sorters are not in cell culture hoods, so if you want to culture your sorted cells it's a good idea to add antibiotics as Pen-strep or gentamycin, and antifungal agents to the collection media. You can also sort directly into lysis buffer (for example, buffer that contains Trizol).

## CAN YOU DETERMINE THE IDEAL SORT CONDITIONS?

We can adjust the **temperature** of the sorters to 4 ºC, 20 ºC, 37 ºC, 42 ºC or at room temperature, and we can keep your sorted cells on ice, if that is what you need.
We need to use **the adequate nozzle** for your experiment.
Our sorters have the following options:

- 70 µm nozzle / 70 psi
- 85 µm nozzle / 45 psi
- 100 µm nozzle / 20 psi

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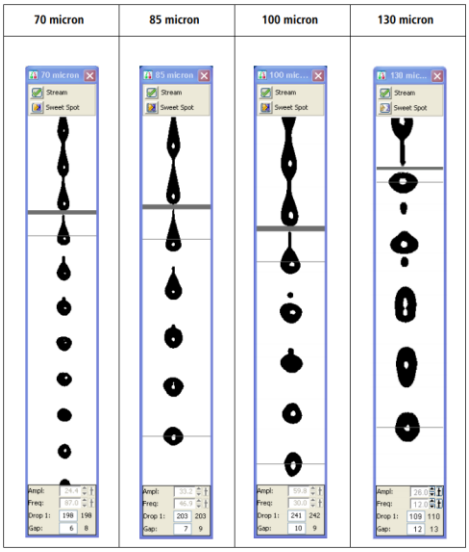
**The nozzle selection depends on the size and sensibility of your cells.**

The most important thing to consider: cells that are fragile or easily stressed (e.g. cultured cells) need a lower pressure sort (100 µm nozzle), independently of their size.

| Nozzle Size (µm) | Sheath Pressure (Psi) | Cell Type |
|---|---|---|
| 70 | 70 | Non-activated T cells<br>B cells<br>Platelets<br>Bacteria<br>Yeast |
| 85 | 45 | Activated T cells<br>Plasma Cells<br>NKT cells<br>NK cells<br>Monocytes<br>mDC<br>pDC<br>Stem cells |
| 100 | 20 | Neurons<br>Macrophages<br>Stem cells<br>Cell lines |




The BD FACSAria sorts faster with a smaller nozzle at higher pressure - 70 µm nozzle. With this nozzle, you can sort up to 20,000 events/second. This nozzle is a great choice for small cells that are happy in single-cell suspension (e.g., blood and bone marrow).

Your cells should be no more than one-third of the size of the nozzle. Therefore, if you have larger cells, they need a larger nozzle size (85 µm or 100 µm nozzle). With these nozzles, sorts are subjected to lower pressures: with the 85 µm nozzle, you can sort at 10,000 events/second, and with the 100 µm nozzle you can sort up to 7,000 events/second.

We normally use PBS (0.9% NaCl) in our sorters as sheath fluid. Each individual cell is sorted inside a tiny drop of this sheath fluid. If you are using the 70 µm nozzle (high pressure, faster sorting), the drops are small and you will get approximately 1 ml of sheath fluid with every 1 million sorted cells. If you are using the 100um nozzle (low pressure, slower sorting), with bigger drops, you will get approximately 3 ml of sheath fluid with every 1 million sorted cells.

| Tubes | Precision mask | Collection volume | Max. number of cells: 70 µm nozzle | 85 µm nozzle | 100 µm nozzle |
|---|---|---|---|---|---|
| 1.5 mL tubes | 4-Way Purity | 250 µL | 1 100 000 | 420 000 | 350 000 |
| | | 500 µL | 800 000 | 320 000 | 200 000 |
| | 0-16-0 | 250 µL | 1 000 000 | | 400 000 |
| | | 500 µL | 800 000 | | 300 000 |
| | Yield | 500 µL | 400 000 | | 100 000 |
| FACStubes | 4-Way Purity | 250 µL | 3 700 000 | 1 600 000 | 1 200 000 |
| | | 500 µL | 3 500 000 | 1 400 000 | 1 000 000 |
| | | 1000 µL | 3 000 000 | 1 200 000 | 800 000 |
| | 0-16-0 | 250 µL | 4 000 000 | 1 600 000 | 1 000 000 |
| | | 500 µL | 3 500 000 | | 800 000 |
| | | 1000 µL | 3 000 000 | | 600 000 |
| | Yield | 1000 µL | 1 700 000 | | 500 000 |
| 15 mL tubes | 4-Way Purity | 500 µL | 12 000 000 | | 4 100 000 |
| | | 1000 µL | 11 000 000 | 5 200 000 | 3 750 000 |
| | | 2000 µL | 10 000 000 | 4 800 000 | 3 500 000 |
| | | 3000 µL | 9 000 000 | 4 400 000 | 3 200 000 |
| | 0-16-0 | 500 µL | 12 000 000 | 5 000 000 | 3 500 000 |
| | | 2000 µL | | 4 200 000 | 3 200 000 |
| | | 3000 µL | | | 3 000 000 |
| | Yield | 1000 µL | 6 000 000 | | 2 000 000 |

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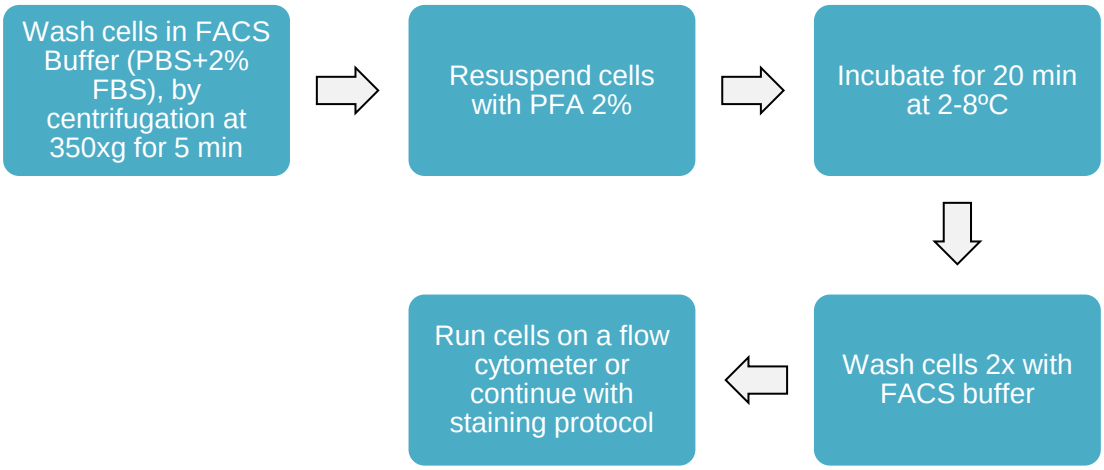
## WHAT SHOULD YOU BRING WITH YOU?

| Sorting Checklist | Analysis Checklist |
|---|---|
| Unstained | Unstained |
| Single colors | Single colors |
| Samples (on ice if appropriate) | Samples (on ice if appropriate) |
| Gating strategy | Gating strategy |
| Collection tubes/plates with 1/3 of media | Extra media/FACS buffer to dilute samples |
| Extra media/FACS buffer to dilute samples | Your DIVA password |

# SOP.UCF.005 – PREPARE SAMPLES FOR FLOW CYTOMETRY

## APPENDIX A - Cell Fixation Protocol




- Samples should not be left in PFA overnight, this may increase the autofluorescence.

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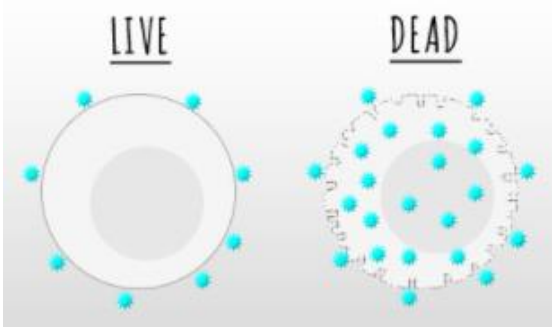
## **Appendix B** - Cell Viability Staining

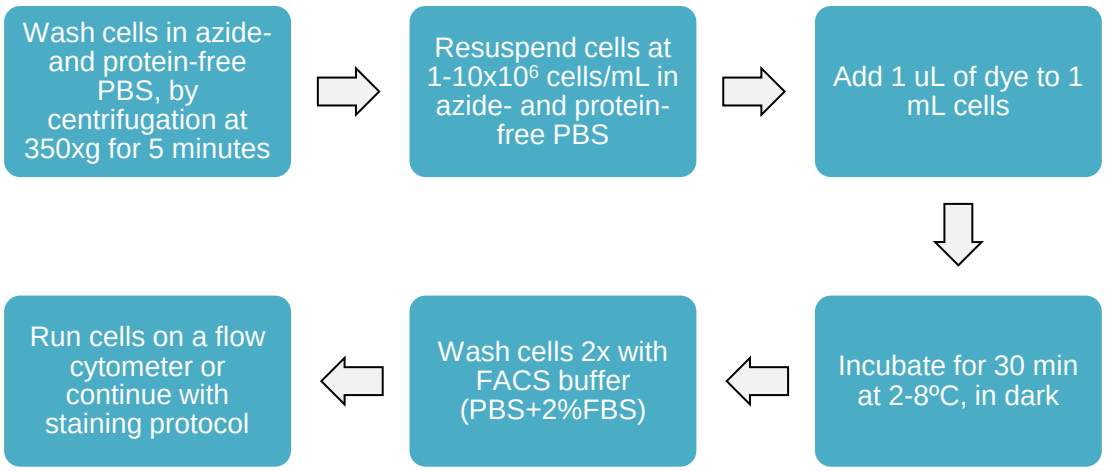
### Fixable Viability Dyes

Fixable dyes have the ability to stain cells and preserve that staining pattern after fixation and/or permeabilization. **If you need to fix your cells afterwards, you should use these dyes!**

The **LIVE/DEAD fixable dead cell stains** are the most commonly known. The staining is based on the reaction of a fluorescent reactive dye with cellular proteins. On live cells, the dye only labels the cell surface proteins, while in dead cells, it can penetrate the damaged membranes and bind to internal proteins, resulting in very bright fluorescence. They are available in multiple single-color formats and compatible with multiple lasers.




It is recommended that experimental conditions, such as incubation time, and temperature, be optimized for each flow cytometry experiment.




- For a brighter staining, it is best to stain in azide- and protein-free PBS;
- Cells may be stained for LD before or after surface staining;
- After this viability staining, cells can be cryopreserved for analysis at a later time;
- This staining can be used in combination with fixation, permeabilization, and intracellular staining. It can also be used in live, unfixed cells;
- To do the single-color sample for compensations, if the percentage of dead cells is less than 5%, it is recommended to take a small aliquot of cells and heat them at 65ºC for 1 min. The heat-killed cells can be combined 1:1 with live cells and then stained with the fixable dye.

Some fixable viability dyes available:

-*Invitrogen*™ LIVE/DEAD™ Fixable Violet, Yellow, Green, and Far Red stain;

-*Biolegend®* Zombie Green, Violet, Yellow and Aqua;

-*Bio-Rad Laboratories, Inc.* VivaFix 410/450, 408/512, 398/550, 498/521, 547/573 and 649/660

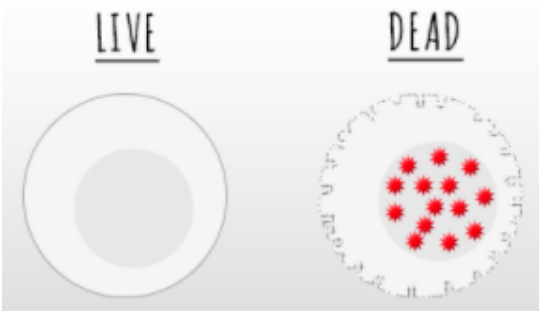
-*BD Biosciences* Horizon FVS 450, 510, 520, 570, 660 and 700.

## Non-Fixable Viability Dyes

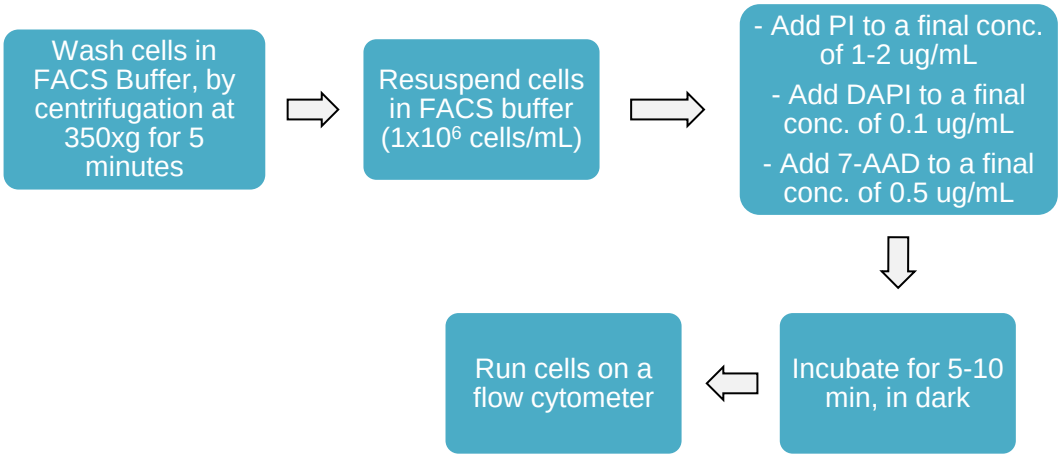
Non-Fixable Viability Dyes are not compatible with fixations. They are high affinity DNA-binding dyes that are effectively excluded from live cells.

The most widely-used are the Propidium Iodide, 7-AAD, DAPI, SYTOX DNA-binding dyes and Annexin V.

It is recommended that experimental conditions, such as incubation time, and temperature, be optimized for each flow cytometry experiment.

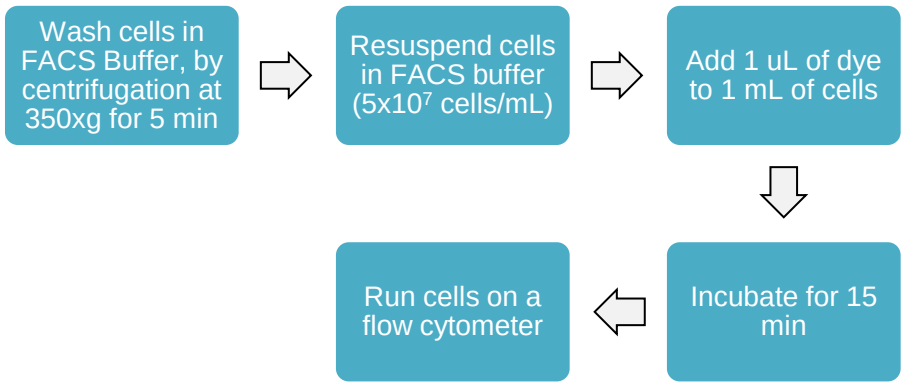



**Propidium Iodide, 7-AAD and DAPI** are cell-impermeant classic DNA-binding dyes. These dyes can only freely enter cells with compromised cell membranes. PI and DAPI intercalate into dsDNA or dsRNA, while 7-AAD will intercalate preferentially with dsDNA. Noncovalent forces mediate these intercalations, reason why the dyes must remain present in the buffer used to resuspend cells for data acquisition so that dead cells will remain labeled.



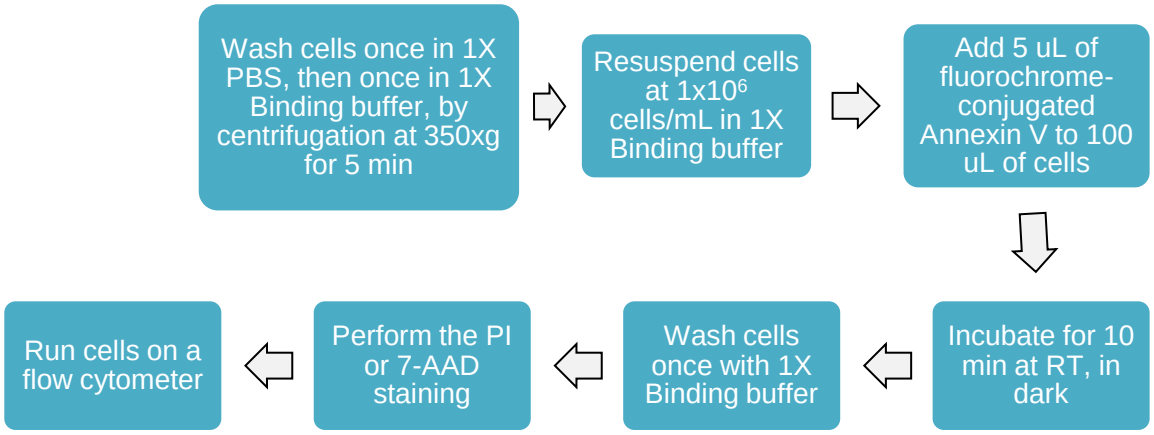

- Cells should be analyzed within 4 hours after staining due to adverse effects on the viability of cells left in the present of PI or 7-AAD for prolonged periods. If required, store at 2-8ºC and protect from light until ready for analysis.
- High concentrations of DAPI may still enter intact cells. Titration must be done with your specific cells, and the incubation time required before cell analysis.

**SYTOX Dead Cell Stains** do not cross intact cell membranes. When entering through damaged membranes of compromised cells they exhibit increased fluorescence upon dsDNA binding. They are non-fluorescent in aqueous media, so they can be added to cells without any additional wash step. They are available in multiple single-color formats and compatible with multiple lasers (*Invitrogen*™ SYTOX™ Blue, SYTOX™ Green, SYTOX™ Orange, SYTOX™ AADvanced™ and SYTOX™ Red).




**Annexin V** is used to detect apoptotic cells. The staining only works in live cells and tissue. It is available in multiple single-color formats and compatible with multiple lasers.

Annexin V has a high affinity for the anionic phospholipid phosphatidylserine (PS). In normal cells, PS is located on the cytoplasmic surface of the plasma membrane. However, during apoptosis, the plasma membrane undergoes structural changes that include translocation of PS from the inner to the outer surface of the plasma membrane.

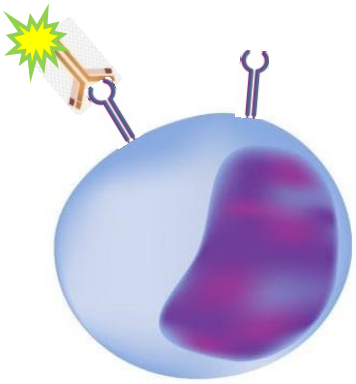



<u>Some available kits allow the simultaneous staining of Annexin and PI, such as:</u>

- *Invitrogen*™ Dead Cell Apoptosis Kit (*ref.* V13242)
- *Abcam* Annexin V-FITC Apoptosis Staining / Detection Kit (*ref.* ab14085)
- *Biolegend* FITC Annexin V Apoptosis Detection Kit with PI (*ref.* 640914)

## Appendix C - Cell Staining

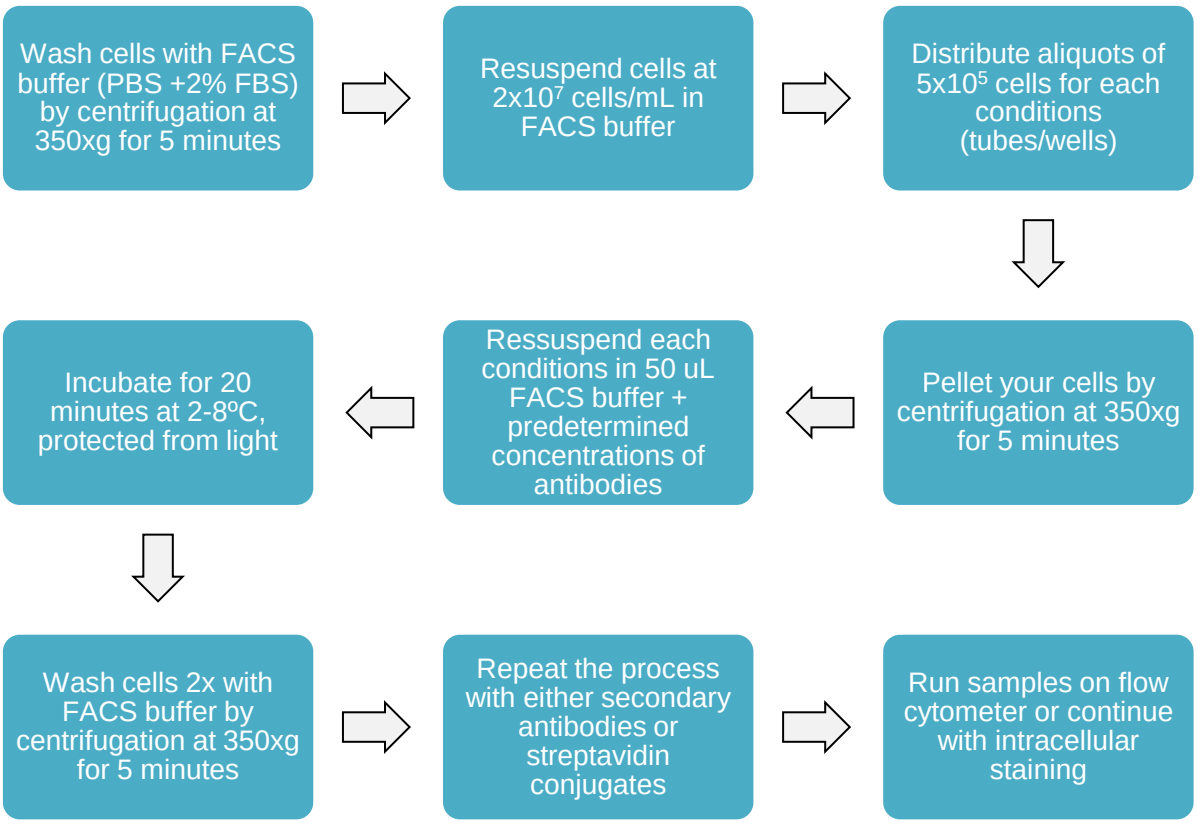
### Surface Staining



Immunophenotyping is a very common flow cytometry test in which fluorophore-conjugated antibodies are used as probes to stain target cells with high avidity and affinity. This technique allows rapid and easy phenotyping of each cell line in a heterogeneous sample according to the presence or absence of a protein combination.

Cell surface proteins such as ion channels, glycoproteins and phospholipids are readily accessible to the antibody so a permeabilization step is not required.

It is recommended that experimental conditions, such as antibody concentration, incubation time, and temperature, be optimized for each flow cytometry experiment.

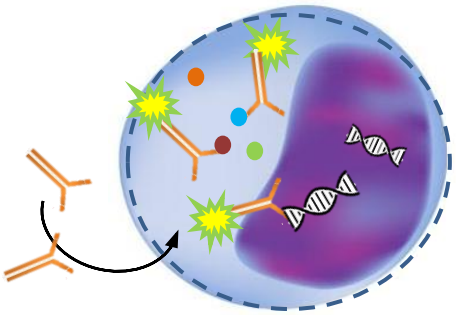



- If analysis must be delayed, you can fix your cells with PFA (Appendix A). However, not all antibodies have been tested for fixations. Verify if the fixation protocol does not affect antibody binding and/or fluorescence intensity.

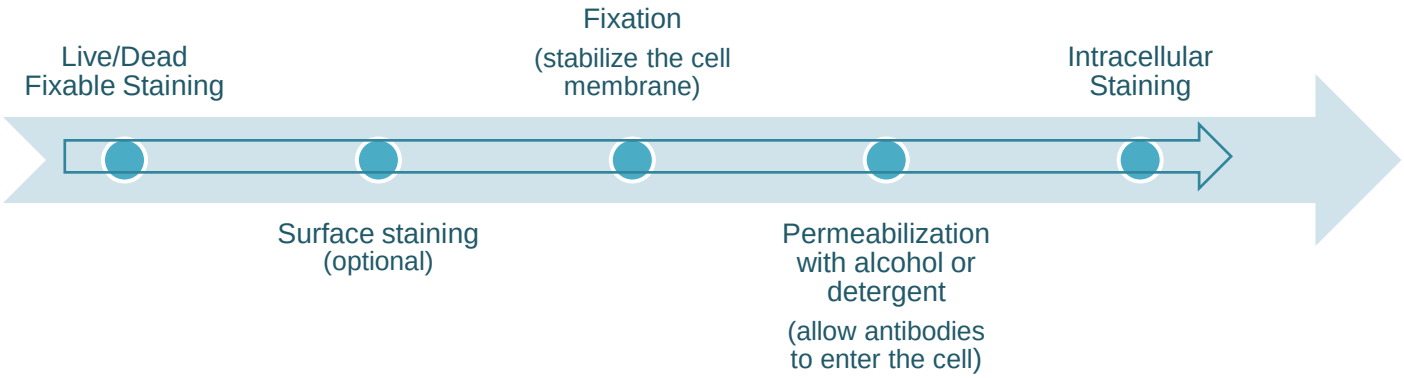
# SOP.UCF.005 – PREPARE SAMPLES FOR FLOW CYTOMETRY

## Intracellular Staining

Intracellular flow cytometry can be used to analyze a variety of intracellular molecules including cytokines, inflammatory mediators, transcription factors and phosphoproteins. It can provide you rich information about the function and signaling responses of specific cell subsets.



The protocol for most intracellular staining experiments, follows the next steps:




There are several ways to permeabilize your cells after fixation:

- With Saponin buffer (PBS/ 0.1% Saponin/ 10% FBS)
- With 0.2% Tween Buffer
- With High Grade Ethanol (-20ºC)

There are also **Commercial buffer kits for Fixation and Permeabilization** that you can easily use to perform your intracellular staining protocol:

- Simultaneous Fixation and Permeabilization

  (one-step protocol; recommended for the detection of intracellular nuclear protein, like Transcription Factors)

  - *BD* Cytofix/Cytoperm™ Fixation/Permeabilization Kit (Cat No. 554714)
  - *Thermo fisher* Foxp3 / Transcription Factor Staining Buffer Set (Cat No. 00-5523-00)
  - *BioLegend* Cyto-Fast™ Fix/Perm Buffer Set (Cat No. 426803)

- Fixation + Permeabilization

  (two-step protocol; recommended for the detection of intracellular cytoplasmic proteins, like Cytokines)

  - *Thermo Fisher* Intracellular Fixation & Permeabilization Buffer Set (Cat. no. 88-8824)
  - *BioLegend* Fixation and Intracellular Staining Perm Wash buffers (Cat. No. 420801 and 421002)

For cytokine and chemokine detection:

- Cells must be stimulated in-vitro (cell culture) or in-vivo (animal/tissue model). Different cytokines/chemokines have different production peaks. In order to obtain optimal staining signals, the stimulation conditions for each stimulant need to be optimized;
- It is critical to include a protein transport inhibitor such as brefeldin A or monensin in the last 4-6 hours of cell culture activation (to block the cytokines inside the cell).

For detection of activated phosphoproteins:

- Cells must be stimulated at 37ºC in RPMI medium with appropriate treatment for desired time points. After this, cells must be immediately fixed in order to maintain phosphorylation state of the protein.

After fixation/permeabilization the intracellular staining is performed mostly as the surface one, but in this case, you will use fixation/permeabilization buffers to dilute your antibodies.

It is recommended that experimental conditions, such as antibody concentration, incubation time, and temperature, be optimized for each flow cytometry experiment.

## DNA Staining

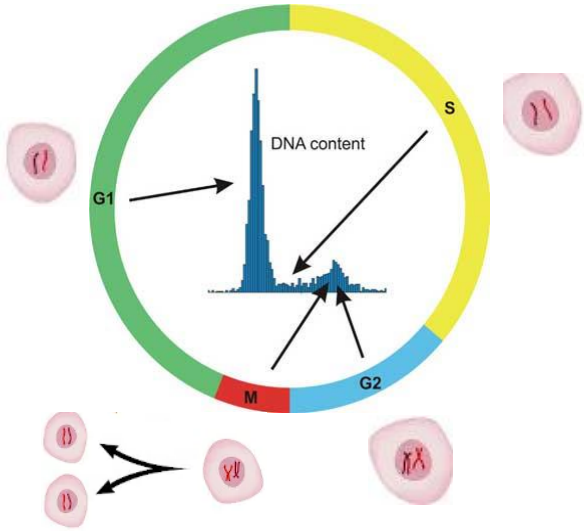
The cell cycle profile of a sample can be determined by staining the DNA with a fluorescent dye and measuring its intensity.

### Cell-impermeant dyes:

For dyes to enter into the cell, these protocols require fixation and permeabilization steps before staining.

**Propidium iodide** or **DAPI** staining of total DNA has been widely adopted in cell cycle analysis.

However, they stain all double-stranded nucleic acids, including dsRNA, so cells must be treated with RNase before staining.




Wash cells 2x in PBS, by centrifugation at 350xg for 5 minutes

→ *(optional)* Perform a PFA fixation (Appendix A) *If you want to retain citosol structure and/or fluorescence (e.g GFP)*

→ Resuspend cells in ice cold PBS at $2x10^6$ cells/mL

→ Add 4ml of 70% pre-chilled -20ºC ethanol (water dilluted) drop by drop to the cell pellet while vortexing

↓ Permeabilize for 1 hour at 4°C

← Wash cells 2x in PBS, by centrifugation at 350xg for 5 minutes

← Resuspend cells at $2x10^6$ cells/mL in PBS

← Treat cells with RNAse at 100µg/ml for 10 min, 37ºC

↓ -Add PI at 20-50µg/ml for 15 min (37ºC) or 30 min (RT)

-Add DAPI at 1-10µg/ml for 15 min (37ºC) or 30 min (RT)

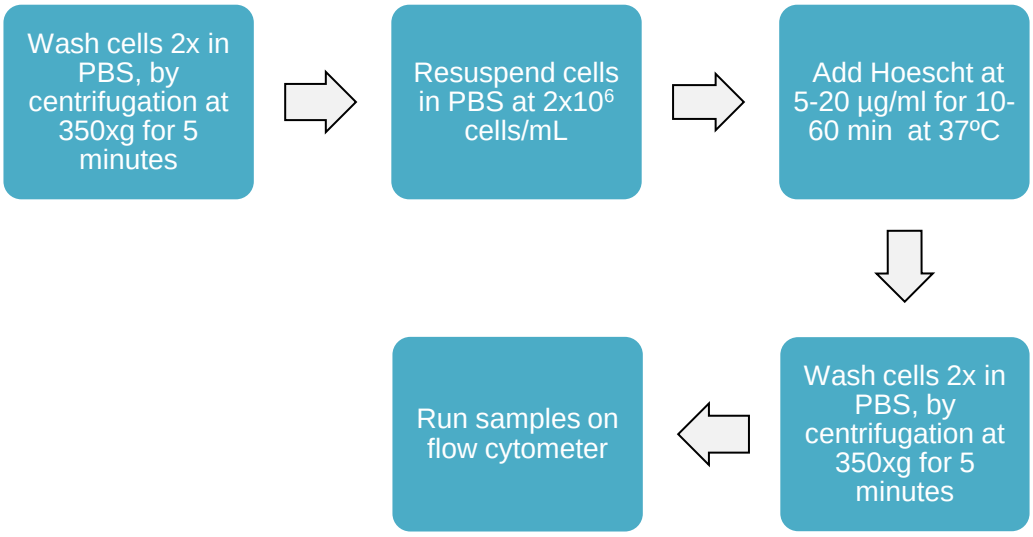
→ Run samples on flow cytometer

- Store cells at 4ºC protected from light.
- Cells should be analyzed within 48 hours after staining.

## Cell-permeant dyes:

These dyes pass freely through the cell membrane, so there is no need for fixation and permeabilization. This is very useful if you want to evaluate cell cycle in your live cells, and recover and grown them afterwards.

**Hoescht 33342** staining has been widely adopted in cell cycle analysis of live cells. In our cytometers, you can excite Hoescht with the Violet laser and you must measure the emitted fluorescence between 425 nm and 480nm.




- The concentration and incubation time of Hoechst used must be pre-determined, as they can vary a lot between different cell types

Besides Hoescht 33342, some dyes like the *ThermoFisher* Vybrant DyeCycle stains were developed to provide a more flexible fluorochrome selection and panel design. They are available for all common laser lines (UV, Violet, Blue, Yellow-Green, and Red) and they can be used to sort cells based on cell cycle staining.